Research Note

# Differential expression of Toll-like receptor pathway genes in chicken embryo fibroblasts from chickens resistant and susceptible to Marek's disease

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**ABSTRACT** The Toll-like receptor (TLR) signaling pathway is one of the innate immune defense mechanisms against pathogens in vertebrates and invertebrates. However, the role of TLR in non-MHC genetic resistance or susceptibility to Marek's disease (MD) in the chicken is yet to be elucidated. Chicken embryo fibroblast (CEF) cells from MD susceptible and resistant lines were infected either with Marek's disease virus (MDV) or treated with polyionosinic-polycytidylic acid, a synthetic analog of dsRNA, and the expression of TLR and pro-inflammatory cytokines was studied at 8 and 36 h posttreatment by quantitative reverse transcriptase PCR. Findings of the present study reveal that MDV infection and polyionosinic-polycytidylic acid treatment significantly elevated the mRNA expression of TLR3, IL6, and IL8 in both susceptible and resistant lines. Furthermore, basal expression levels in uninfected CEF for *TLR3*, *TLR7*, and *IL8* genes were significantly higher in resistant chickens compared with those of susceptible chickens. Our results suggest that *TLR3* together with pro-inflammatory cytokines may play a significant role in genetic resistance to MD.

**Key words:** Marek's disease, resistance, Toll-like receptor, cytokine

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## INTRODUCTION

Marek's disease (**MD**) is a lymphoproliferative disease of chicken caused by *Gallid herpesvirus 2* (an alphaherpesvirus), which is also known as Marek's disease virus (**MDV**). This disease affects birds at a very young age and causes severe economic losses due to reduced egg production in layers (egg-type chickens) and reduction in growth rate and condemnation of meats in broilers (meat-type chickens). Because the virus is immunosuppressive, birds affected with this disease are also vulnerable to other secondary infections. Genetic resistance to MD has been well established with the best understood locus being the MHC (Briles et al., 1977). However, other genetic factors are known to exist that have a major influence in MD resistance as exemplified by Avian Disease and Oncology Laboratory lines 6 and 7, which both share the same B2 MHC haplotype, yet are genetically resistant and susceptible, respectively (Bacon et al., 2001; Cheng et al., 2013).

Toll-like receptors (**TLR**) are a class of proteins that recognize molecules shared by pathogens and play a significant role in innate immune competence through the induction of cytokines via the TLR signaling pathway. Expression of TLR and IL in response to MDV infection in chicken has been previously reported with expression of TLR3 and TLR7 in lung and spleen up- and downregulated, respectively, following viral infection (Abdul-Careem et al., 2009; Jie et al., 2013). With regard to interleukins, expression of IL1β, IL6, and IL8 was upregulated in MDV-infected chickens (Abdul-Careem et al., 2009; Jie et al., 2013). Significant upregulation of IL6 and IL18 in MD susceptible, and IL1β and IL8 in MD resistant chicken lines was also reported following MDV infection (Kaiser et al., 2003). Recently, differential expression and allele specific expression analysis of genes upon MDV challenge using RNA-seq data identified 2 immunological pathways: TLR signaling and Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathways (Perumbakkam et al., 2013). Therefore, a study was undertaken with the objectives of studying the expression of select genes of the TLR signaling pathway in response to MDV infection or treatment with polyionosinic-polycytidylic acid [**poly (I:C)**] in cultured chicken embryo fibroblast (**CEF**) cells from MD resistant and susceptible chickens.

## MATERIALS AND METHODS

### Cells and Virus

Chicken embryo fibroblasts (**CEF**) obtained from six d-10 embryos were prepared and pooled from each of


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Avian Disease and Oncology Laboratory line $6_3$ (MD resistant) and line $7_2$ (MD susceptible), and secondary cultures plated at a density of $4 \times 10^6$ cells per 100-mm dish. Cells were cultured in Leibowitz's L-15 and McCoy 5A media with 4% heat inactivated fetal bovine serum, amphotericin (2 μg/mL), penicillin (20,000 U/mL), and streptomycin (20 μg/mL), and maintained at 37°C with 5% $CO_2$. Secondary CEF were grown up to 24 h or until the appearance of a complete monolayer in 100-mm plates before proceeding with the infection or treatment. The MDV generated from Md5B40BAC1, our infectious BAC clone that contains the Md5 strain MDV genome and generates virulent MDV, was used for infection (Niikura et al., 2011).

### Experimental Design

For MDV-treated cells, CEF in 100-mm plates were infected with 30,000 pfu MDV per plate. As a control for induction of gene expression and to compare between MD resistant and susceptible CEF, cells were treated with 50 μg/mL poly (I:C), a synthetic analog of dsRNA (InvivoGen, San Diego, CA), 3 h before the first infection time point (8 h postinfection or hpi). Untreated CEF cells were considered as negative controls. Experiments involving technical replicates of each group were done in triplicate.

### Extraction of RNA and cDNA Synthesis

Total RNA from CEF was extracted at 8 and 36 hpi, which reflect early and late viral infection time points, using a total RNA isolation mini kit (Agilent Technologies, Santa Clara, CA) per the recommendations of the manufacturer. The quality and concentration of RNA was tested using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA). Subsequently 500 ng of RNA was reverse transcribed to synthesize cDNA through random primers using the high capacity cDNA RT kit (Applied Biosystems Inc., Foster City, CA) as per the manufacturer's instructions.

### Quantitative PCR

Gene expression levels were measured using Power SYBR master mix (Applied Biosystems Inc.) on an ABI 7500 Real-Time PCR System with 96-well plates. The glyceraldehyde 3-phosphate dehydrogenase (***GAPDH***) gene was used for normalization, and each sample was analyzed in duplicate. Forward and reverse primers for each gene were synthesized by Eurofins MWG Operon (Huntsville, AL) and used at 0.4 μ*M*: *TLR3*, TCAGTACATTTGTAACACCCCGCC and GGCGTCATAATCAAACACTCC; *TLR4*, TGCCATCCCAACCCAACCACAG and ACACCCACTGAGCAGCACCAA; *TLR7*, TTCTGGCCACAGATGTGACC and CCTTCAACTTGGCAGTGCAG; *IL1β*, GTGAGGCTCAACATTGCGCTGTA and TGTCCAGGCGGTAGAAGATGAAG; *IL6*, CGTGTGCGAGAACAGCATGGAGA and TCAGGCATTTCTCCTCGTCGAAGC; and *IL8*, CCAAGCACACCTCTCTTCCA and GCAAGGTAGGACGCTGGTAA. The PCR conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and combined annealing/extension at 60°C for 60 s. A melting curve analysis at the end of each reaction was performed to confirm the presence of a single amplification product. Relative gene expression (**ΔCt**) was calculated by normalizing target gene Ct values with those of the endogenous control, *GAPDH* gene. The ΔCt values of each sample were subtracted from 40 to calculate 40−ΔCt values; higher numerical values of 40−ΔCt reflect higher mRNA expression (MacKinnon et al., 2009). Fold change in mRNA expression was calculated as $2^{-\Delta\Delta Ct}$ between resistant and susceptible lines within each treatment. Fold change difference was also calculated between treatments within lines. Mean values of 40−ΔCt and $2^{-\Delta\Delta Ct}$ were calculated to compare between lines and treatments. Student's *t*-test was used for testing significant difference in the expression of genes between CEF from susceptible and resistant lines and one-way ANOVA to test the difference between treatments (infected, treated, and uninfected CEF cells) at the 2 time points. Differences were considered significant at $P \leq 0.05$.

To monitor viral copy number in MDV-infected cells, MDV UL27, which encodes gB, were compared with chicken GAPDH using the method as described by Gimeno et al. (2008).

## RESULTS AND DISCUSSION

Understanding the genetic basis of MD resistance would be useful in developing breeding strategies for control of this disease. The role of innate immune competence in MD resistance through study of expression of TLR signaling pathway genes was investigated using CEF from MD resistant and susceptible lines. Admittedly, CEF are not the natural target cells for MDV; however, there is a high degree of overlap when comparing results from other studies that use birds (MacEachern et al., 2011; Smith et al., 2011; Yu et al., 2011; Perumbakkam et al., 2013) with those derived from cells (Subramaniam et al., 2013), suggesting that CEF are reasonable models. Expression of TLR3 was significantly higher in MDV-infected and poly (I:C) treated CEF compared with uninfected CEF in both MD susceptible and resistant lines at the 2 times monitored (Tables 1 and 2; a higher number indicates higher expression levels); no significant differences were observed in viral copy number between CEF from the 2 chicken lines (data not shown). Previously it was demonstrated that expression of TLR3 is upregulated in lung tissues (Abdul-Careem et al., 2009) and bursa (Jie et al., 2013) following MDV infection in chickens. Thus, our results

**Table 1.** Differential expression of mRNA (40−ΔCt) of Toll-like receptor (*TLR*) and interleukin (*IL*) genes in chicken embryo fibroblasts (CEF) of resistant and susceptible lines at 8 h postinfection (hpi)

| Gene name | Treatment[1] | Resistant line | Susceptible line | *P*-value |
|---|---|---|---|---|
| *TLR3* | Uninfected | 30.0 ± 0.05[c] | 28.9 ± 0.16[c] | <0.001 |
| | Infected | 31.5 ± 0.13[b] | 30.4 ± 0.09[b] | <0.001 |
| | Poly (I:C) | 35.2 ± 0.18[a] | 34.5 ± 0.03[a] | <0.01 |
| | *P*-value | <0.0001 | <0.0001 | — |
| *TLR4* | Uninfected | 27.8 ± 0.36[b] | 26.9 ± 0.44 | 0.137 |
| | Infected | 27.5 ± 0.20[b] | 27.2 ± 0.27 | 0.223 |
| | Poly (I:C) | 29.8 ± 0.13[a] | 28.2 ± 0.27 | <0.01 |
| | *P*-value | <0.001 | 0.092 | — |
| *TLR7* | Uninfected | 29.5 ± 0.25 | 28.0 ± 0.36 | <0.01 |
| | Infected | 29.4 ± 0.29 | 28.1 ± 0.18 | <0.01 |
| | Poly (I:C) | 29.9 ± 0.34 | 28.1 ± 0.16 | <0.01 |
| | *P*-value | 0.899 | 0.599 | — |
| *IL1β* | Uninfected | 24.5 ± 0.09[b] | 24.5 ± 0.21[b] | 0.437 |
| | Infected | 24.6 ± 0.23[b] | 24.7 ± 0.26[b] | 0.397 |
| | Poly (I:C) | 31.1 ± 0.53[a] | 30.1 ± 0.35[a] | 0.105 |
| | *P*-value | <0.001 | <0.001 | — |
| *IL6* | Uninfected | 32.1 ± 0.09[b] | 31.7 ± 0.15[b] | 0.055 |
| | Infected | 32.2 ± 0.20[b] | 31.7 ± 0.14[b] | <0.05 |
| | Poly (I:C) | 36.4 ± 0.15[a] | 35.8 ± 0.09[a] | <0.01 |
| | *P*-value | <0.0001 | <0.0001 | — |
| *IL8* | Uninfected | 35.1 ± 0.28[b] | 32.0 ± 0.26[c] | <0.001 |
| | Infected | 36.0 ± 0.25[b] | 33.3 ± 0.20[b] | <0.001 |
| | Poly (I:C) | 40.9 ± 0.46[a] | 39.0 ± 0.11[a] | <0.01 |
| | *P*-value | <0.001 | <0.0001 | — |

[a–c]Different superscripts within a column for each gene denote values that are significantly different from each other.

[1]Poly (I:C) = treatment with polyionosinic-polycytidylic acid.

are in agreement and extend the importance of TLR3 as its expression was significantly higher in the resistant line compared with the susceptible line.

It was also interesting to note that expression of TLR3 in infected and poly (I:C) treated and its basal level in uninfected CEF from the resistant line were strikingly higher relative to the susceptible line, suggesting that genetic selection for MD resistance enhances expression of the *TLR3* gene even in uninfected cells, and this feature might be responsible for better protection of resistant chickens against MD. Expression of TLR3 in poly (I:C) treated cells was much more

**Table 2.** Differential expression of mRNA (40−ΔCt) of Toll-like receptor (*TLR*) and interleukin (*IL*) genes in chicken embryo fibroblasts (CEF) of resistant and susceptible lines at 36 h postinfection (hpi)

| Gene name | Treatment[1] | Resistant line | Susceptible line | *P*-value |
|---|---|---|---|---|
| *TLR3* | Uninfected | 29.8 ± 0.19[c] | 26.5 ± 0.29[c] | <0.01 |
| | Infected | 30.7 ± 0.34[b] | 29.0 ± 0.09[b] | <0.01 |
| | Poly (I:C) | 33.9 ± 0.14[a] | 32.9 ± 0.19[a] | <0.01 |
| | *P*-value | <0.0001 | <0.0001 | — |
| *TLR4* | Uninfected | 26.7 ± 0.56[b] | 25.9 ± 0.45 | 0.172 |
| | Infected | 27.2 ± 0.22[b] | 26.4 ± 0.68 | 0.176 |
| | Poly (I:C) | 28.9 ± 0.20[a] | 27.1 ± 0.07 | <0.001 |
| | *P*-value | <0.05 | 0.319 | |
| *TLR7* | Uninfected | 29.9 ± 0.04[b] | 27.4 ± 0.12[b] | <0.0001 |
| | Infected | 30.1 ± 0.03[a] | 27.4 ± 0.001[b] | <0.0001 |
| | Poly (I:C) | 30.3 ± 0.08[a] | 28.3 ± 0.29[a] | <0.01 |
| | *P*-value | <0.05 | <0.05 | |
| *IL1β* | Uninfected | 25.1 ± 0.25[b] | 24.5 ± 0.17[b] | 0.051 |
| | Infected | 24.5 ± 0.26[b] | 24.4 ± 0.08[b] | 0.410 |
| | Poly (I:C) | 33.1 ± 0.23[a] | 32.2 ± 0.33[a] | 0.052 |
| | *P*-value | <0.001 | <0.001 | — |
| *IL6* | Uninfected | 31.7 ± 0.50[b] | 32.2 ± 0.06[b] | 0.186 |
| | Infected | 32.6 ± 0.09[b] | 32.3 ± 0.06[b] | <0.05 |
| | Poly (I:C) | 36.0 ± 0.31[a] | 35.3 ± 0.08[a] | <0.01 |
| | *P*-value | <0.0001 | <0.0003 | — |
| *IL8* | Uninfected | 34.7 ± 0.38[c] | 31.1 ± 0.12[b] | <0.001 |
| | Infected | 35.3 ± 0.33[b] | 32.4 ± 0.11[b] | <0.001 |
| | Poly (I:C) | 41.2 ± 0.46[a] | 38.7 ± 0.15[a] | <0.01 |
| | *P*-value | <0.001 | <0.0001 | — |

[a–c]Different superscripts within a column for each gene denote values that are significantly different from each other.

[1]Poly (I:C) = treatment with polyionosinic-polycytidylic acid.

prominent compared with that of infected cells in resistant and susceptible lines. It is established that poly (I:C), a synthetic analog of dsRNA, stimulates TLR3 expression in spleen and DF1 cells (Karpala et al., 2008) and it has also been reported to provide protection as an adjuvant against virulent strains of MDV in HVT-vaccinated chickens (Parvizi et al., 2012). These observations support the finding that poly (I:C) provides protection against MD in chicken likely through upregulation of TLR3.

No effect of infection or poly (I:C) treatment on expression of TLR7 mRNA was observed at 8 hpi (Table 1). However, differences were observed in both lines following MDV infection, poly (I:C) treatment, or both at 36 hpi (Table 2). It was reported that expression of TLR7 was upregulated in lung and spleen at 14 dpi, and downregulated at 28 dpi in spleen following MDV infection (Abdul-Careem et al., 2009; Jie et al., 2013). Taken together, it is possible that a higher basal level of TLR7 may be associated with increased MD resistance.

No significant difference was observed in expression of TLR4 between infected and uninfected CEF in both susceptible and resistant lines at both time points. However, expression of TLR4 in poly (I:C) treated cells was significantly higher than uninfected CEF in both lines at both time points. Similarly, there were no significant differences between resistant and susceptible lines in infected and uninfected cells, whereas its expression in poly (I:C) treated cells of the resistant line was significantly higher than that of the susceptible line (Tables 1 and 2). The TLR4 is reported to be expressed in CEF cells (Iqbal et al., 2005), and is mainly involved in bacterial infections as lipo-polysaccharide is recognized as its ligand. Hence, there was a lack of significant difference in expression of TLR4 gene between MDV-infected with uninfected cells and also between MD susceptible and resistant lines. Differential and allele specific expression of the *TLR4* gene in response to MD infection was observed in $F_1$ progeny of resistant and susceptible layer chickens (Perumbakkam et al., 2013). In contrast, we could see no differences between infected and uninfected cells, and also between susceptible and resistant CEF in TLR4 expression although there was an effect of poly (I:C) treatment on its expression. Therefore, further study in live animals of MD resistant and susceptible lines is required to establish the role, if any, of TLR4 in MD resistance.

Expression of the *IL8* gene was significantly higher in MDV-infected and poly (I:C) treated CEF compared with uninfected CEF in both MD resistant and susceptible lines at both time points. Its expression was significantly higher in MDV-infected, poly (I:C) treated and uninfected (basal level) CEF from the MD resistant line in comparison with the susceptible line at 8 and 36 hpi. Expression of IL6 was significantly higher in MDV-infected and poly (I:C) treated cells compared with the uninfected cells of both MD resistant and susceptible lines at 8 and 36 hpi. Expression of IL6 in MDV-infected and poly (I:C) treated cells of MD resistant line was significantly higher than susceptible line at 8 and 36 hpi. No significant difference was observed in expression of IL1β between MDV-infected and uninfected CEF in both MD susceptible and resistant lines at both time points. Similarly, there were no significant differences between MD resistant and susceptible lines in MDV-infected cells. However, expression of IL1β in poly (I:C) treated cells was significantly higher than uninfected CEF in both lines at both time points.

Pro-inflammatory cytokines play key role in host defense against viral infections through inflammation and antiviral effects. The IL1β, IL6, and IL8 are downstream products elicited in the TLR signaling pathway. In the present study, it was observed that the expression of IL8 was correlated with the expression of TLR3. Abdul-Careem et al. (2009) reported that expression of TLR3 and that of IL8 and IL1β were significantly correlated in lung tissues of MDV-infected chickens. Therefore, it may be speculated that expression of TLR3 regulates the expression of the above IL in MDV-infected birds.

Among the IL tested for expression in CEF from MD resistant and susceptible lines, IL8 seems to play major role in genetic resistance to MD as their expression in infected as well as poly (I:C) treated cells was significantly upregulated besides being highly induced in the resistant line compared with the susceptible line. These findings indicate that susceptibility to MD is due in part to lesser inflammatory response through lesser expression of these cytokines as seen in the MD susceptible line. It was reported that IL8 and IL1β genes were upregulated in MD resistant (MHC B21 haplotype) birds (Jarosinski et al., 2005). On the contrary, expression of IL6 and IL18 was upregulated in genetically susceptible chickens with no expression of these cytokines in MD resistant lines (Kaiser et al., 2003). However, Parvizi et al., (2009) could not establish an association between cytokine (IL18 and IL6) gene expression in T cell subsets and MHC resistant (B21) or susceptible (B19) chickens to MD. Further, Heidari et al. (2010) reported upregulation of IL6 at 5 d (lytic phase) and downregulation of IL8 and IL18 at 15 d (latent phase) in $F_1$ progeny of $15I_5 \times 7_1$ susceptible chicks following MDV infection in splenocytes. These differences in expression of IL between different studies could be attributed to the fact that expression of IL varies depending upon the genetic background of birds, virulence of MD virus, interaction of pathotype of virus and the genotype of host, and tissues or cells types studied (Schat and Nair, 2008; Haq et al., 2013). Our findings are consistent with the observations of Jarosinski et al. (2005) with regard to expression of IL8 though the chicken lines and MHC haplotype differ.

It was interesting to note that effect of poly (I:C) treatment on expression of interleukins (IL1β, IL6, and IL8) was more prominent in both MD resistant and susceptible chickens in relation to the uninfected and MDV-infected cells and expression of these cytokines being significantly higher in the resistant line (Tables 1

**Table 3.** Fold-change differential expression ($2^{-\Delta\Delta Ct}$) of mRNA of Toll-like receptor (*TLR*) and interleukin (*IL*) genes in the resistant line relative to the susceptible line at 8 and 36 h postinfection (hpi)

| Gene name | Uninfected | Infected | Poly (I:C)[1] |
|---|---|---|---|
| 8 hpi | | | |
| *TLR3* | 2.29 ± 0.36*** | 2.10 ± 0.21*** | 1.71 ± 0.16** |
| *TLR4* | 1.36 ± 0.33 | 1.30 ± 0.30 | 3.15 ± 0.70** |
| *TLR7* | 3.26 ± 0.91** | 2.62 ± 0.52** | 3.41 ± 0.46** |
| *IL1β* | 1.04 ± 0.10 | 0.05 ± 0.004 | 1.96 ± 0.25 |
| *IL6* | 1.31 ± 0.21 | 1.51 ± 0.27* | 1.59 ± 0.23** |
| *IL8* | 9.64 ± 3.22*** | 6.70 ± 0.77*** | 3.96 ± 0.91** |
| 36 hpi | | | |
| *TLR3* | 11.1 ± 3.47** | 3.26 ± 0.57** | 2.02 ± 0.49** |
| *TLR4* | 1.38 ± 0.06 | 2.04 ± 0.92 | 3.69 ± 0.45*** |
| *TLR7* | 5.67 ± 0.31*** | 7.67 ± 0.94*** | 3.95 ± 0.65** |
| *IL1β* | 1.67 ± 0.43 | 0.04 ± 0.007 | 1.81 ± 0.13 |
| *IL6* | 0.80 ± 0.25 | 1.20 ± 0.09* | 1.64 ± 0.26** |
| *IL8* | 13.8 ± 3.76*** | 7.21 ± 1.27*** | 5.95 ± 1.36** |

Significant differences are indicated with $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

[1]Poly (I:C) = treatment with polyionosinic-polycytidylic acid.

and 2). Perhaps poly (I:C) is more readily available for stimulation of TLR3, or the dose used is more potent as it was reported that 5 μg per mL of poly (I:C) induced the upregulation of TLR3 in bursal cells (St. Paul et al., 2012). It was also reported that poly (I:C) treatment significantly enhances the induction of IL6 and IL1β in splenocytes (Villanueva et al., 2011). Poly(I:C) is known to stimulate pro-inflammatory cytokine production through dendritic cell maturation and hence administration of poly (I:C) provides better protection against MD in HVT-vaccinated chicken (Parvizi et al., 2012). To our knowledge, this study is first of its kind to see the effect of poly (I:C) on cytokine expression in CEF from MD resistant and susceptible chickens.

Fold change differential expression of mRNA of TLR and IL genes in the resistant line in relation to the susceptible line was positive and significant at both time points except for *IL1β*, *IL6* (infected cells), and *TLR4* (uninfected and infected cells) genes at both time points (Table 3). There was no significant difference in fold change differential expression of TLR and IL genes in response to infection or poly (I:C) treatment both at 8 and 36 hpi between resistant and susceptible lines except for *TLR3* and *IL6* genes. Fold change differential expression of the *TLR3* gene in response to infection or poly (I:C) treatment was significantly higher in the susceptible line at 36 hpi, whereas the fold change expression of the *IL6* gene was significantly higher in the resistant line at 36 hpi. In conclusion, our findings suggest that TLR3 plays an important role in host response to MDV infection and in genetic resistance to MD together with the upregulation of cytokines, especially IL6 and IL8.

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